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## METHOD OF TREATING A WOUND THEREFOR

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Use of lipopeptides or lipoproteins for wound treatment and prophylaxis of infections

A

State of the Art

Five stages of wound healing can be described:

1. Blood coagulation or release of mediators from thrombocytes (after some minutes);
2. Flowing in of leucocytes, i. e. granulocytes at the beginning, thereafter of macrophages and lymphocytes (day 1 to 3);
3. Proliferation of diverse cells such as fibroblasts, endothelial and epithelial cells (day 3 to 7);
4. Wound contraction (day 7 to 9); and
5. Rearrangement of scar tissue (up to one year).

The flowing in of granulocytes in stage 2 results in the incorporation of debris and the destruction of infectious germs, which is an object, which is also solved by macrophages. In addition, the macrophages are a source of a series of mediators such as signal peptides, growth factors and cytokines, such as Transforming Growth Factor (TGF $\beta$ 1), Platelet Derived Growth Factor (PDGF-AA and -BB), Fibroblast Growth Factor (FGF2) and of TGF- $\alpha$  belonging to the family of Epi-dermal Growth Factors (EGF). Macrophages also secrete interleukin-1 (IL-1) which indirectly induces FGF7 in fibroblasts. All these factors take part in different stages of wound healing or are indispensable therefor. Without macrophages as a source thereof wound healing is considerably delayed or impossible.

#### **Problems**

Despite of the fact that it is possible to use some of the above-mentioned mediators individually for wound healing, this is, however, not effective since most of these peptides have half-live periods of merely some minutes. Additional difficulties reside in the fact that the natural moment when the different mediators appear, the optimal dosage and the interaction of these substances is not known in detail and can much less be checked during applications.

A complication also of surgical wounds may reside in infections which in general result in a delayed wound healing and an increased formation of scars. This is especially problematical in cosmetic surgery. A prophylactic treatment by covering with antibiotica is to a far extent no longer usual in

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view of resistance problems and possible allergic reactions. For certain groups of patients, such as diabetics and older patients, wound healing is delayed.

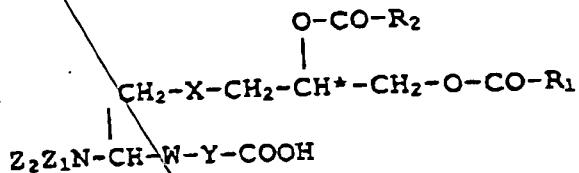
### **Solution**

It is a natural property of mycoplasmas that they lead to a flowing in of leucocytes at the infection site, such as the lungs. We have now been able to show that this property is associated with the presence of a specific class of lipopeptides, which is characterized in that it is provided N-terminally with a dihydroxypropyl-cystein group with two ester-like linked long-chain fatty acids (6, 10). In addition to the already mentioned property, such lipopeptides stimulate murine and human macrophages or monocytes in vitro for the release of cytokines and prostaglandines (1 to 3; 11), and in addition as we were to discover, are able to induce in vivo high titers of the chemokines MIP-1 $\alpha$ , MIP-2, MCP-1 as well as KC and to lead to the flowing in of leucocytes (12). These lipopeptides stimulate the cells via the toll-like receptor 2 (13).

In animal and human medicine, pure and synthetically produced lipopeptides and lipopeptides, which have been incorporated in liposomes or are coupled to biologically degradable polymer carrier substances, can be used e.g. in the form of ointments, lotions or injection solutions. When applied to wounds or injected in the surrounding of wounds, such preparations should increase and accelerate the natural flowing in of granulocytes and macrophages and, as a consequence, prevent infections and facilitate and accelerate the wound healing by

stimulating the biosynthesis of mediators involved in the wound healing in their natural sequence and concentration. The in vivo activity of such lipopeptides from mycoplasmas is surprising and new in view of the fact that the application of different bacterial lipopeptides and their synthetic analogs does not show any effect in animal assays (8).

According to one embodiment the problem underlying the invention is solved by the use of a lipopeptide or lipoprotein of the following general formula



wherein

$R_1$  and  $R_2$ , which may be identical or different from each other, mean a  $C_{7-25}$ -alkyl,  $C_{7-25}$ -alkenyl or  $C_{7-25}$ -alkinyl,  $X$  means S, O or  $\text{CH}_2$ ,

$Z_1$  and  $Z_2$ , which may be identical or different from each other, mean H or methyl,

$W$  means CO or  $S(O)_n$  (for  $n = 1$  or 2) and

$Y$  means a physiologically acceptable amino acid sequence consisting of 1 to 25 amino acid residues and the asymmetric carbon atom marked with \* has the absolute S-configuration if  $X = S$  (sulfur)

for the production of a pharmaceutical preparation for wound treatment of animals or humans.

According to the invention the lipopeptide or lipoprotein can be characterized in that Y means a physiologically acceptable amino acid sequence consisting of 1 to 25 amino acids.

According to the invention the lipopeptide or lipoprotein can be characterized in that Y means an amino acid sequence which is selected from the following group:

- (i) amino acid sequence which does not impair the water solubility of the lipopeptide or lipoprotein.
- (ii) GQTNT
- (iii) SKKKK
- (iv) GNNDESNISFKEK
- (v) GQTDNNSSQSQQPGSGTTNT

whereby in the amino acid sequences (ii), (iii), (iv) and (v) individual amino acids may be omitted or exchanged.

According to the invention the C<sub>7-25</sub>-alkyl, C<sub>7-25</sub>-alkenyl or C<sub>7-25</sub>-alkinyl can be C<sub>15</sub>-alkyl, C<sub>15</sub>-alkenyl or C<sub>15</sub>-alkinyl.

According to the invention the double bond(s) of the C<sub>7-25</sub>-alkenyl residue can have cis-configuration.

According to another embodiment the object underlying the invention is solved by the use of a physiologically acceptable lipopeptide or lipoprotein, which is provided N-terminally with a dihydroxy-propyl-cystein group with two ester-like linked optionally long-chain fatty acids which may be identical or different, for the production of a preparation for wound treatment of animals or humans.

Further, the invention concerns the use of a lipopeptide or lipoprotein obtainable from a mycoplasma clone for the production of a pharmaceutical preparation for wound treatment of animals or humans.

This use may be characterized in that the lipopeptide or lipoprotein is obtainable from a Mycoplasma fermentans clone.

For the use according to the invention the lipopeptide or lipoprotein can be water-soluble or amphoteric.

According to the invention a lipopeptide or lipoprotein selected from the following group may be used:

- (i) S-[2,3-bispalmitoyloxy-(2RS)-propyl]cysteinyl-GQTNT
- (ii) S-[2,3-bispalmitoyloxy-(2RS)-propyl]cysteinyl-SKKKK
- (iii) S-[2,3-bispalmitoyloxy-(2RS)-propyl]cysteinyl-GNNDESNISFKEK
- (iv) S-[2,3-bispalmitoyloxy-(2S)-propyl]cysteinyl-GNNDESNISFKEK
- (v) S-[2,3-bispalmitoyloxypropyl]cysteinyl-GQTDNNSSQSQQPGSGTTNT.

For use according to the invention the lipopeptide or lipoprotein can be provided in form of a solution for epicutaneous application, an injection solution, an ointment, a lotion, an aqueous suspension, a patch impregnated or coated therewith, encapsulated in liposomes or coupled to carrier polymers which are biologically degradable.

As regards the use according to the invention the wounds may be wounds resulting from lesions or surgery, chronically in-

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fected wounds, burns, chronical ulcera or ulcus venosum or wounds of patients who are corpulent or diabetics or have been subjected to a radiation therapy or chemotherapy.

Synthetic lipopeptide preparations could be prepared at an acceptable cost level, and they would increase the natural process of wound healing without principally interfering the complicated control mechanism of the different mediators of the wound healing. In addition, an infection prophylaxis would result without any need of applying antibiotica or other bacteriostatica which e.g. might impair wound healing. Such lipopeptide preparations would also be applicable for face wounds, wherein care should be taken in view of a risk of contacting eyes or the nasal or oral areas in view of the toxicity of usual bactereostatica. When topically applied, the risk of systemic effects such as fever is practically excluded.

The invention is subsequently illustrated by figures and examples.

**Example 1:** Macrophage activation by synthetic lipopeptides which are derived from mycoplasmas as measured by a nitrogen monooxide release assay

The release of nitrogen monooxide from peritoneal exudate cells in the presence of interferon- $\gamma$  is an easily quantifiable assay for the activation of murine macrophages by lipopeptides; cf. e. g. Mühlradt & Frisch in 2. This assay is conducted with peritoneal exudate cells of C3H/HeJ mice which only slightly react with endotoxin in microtiter plates (96 wells).  $10^5$  cells are simultaneously stimulated by rIFN- $\gamma$  and

a series of dilutions of a material which activates macrophages. After an incubation time of 48 hours the nitrate is reduced using nitrate reductase; NO is determined as the sum of nitrite and nitrate using Griess reagent.

The effect of macrophage stimulation of the following lipopeptides is compared in **Figures 1A to 1D**.

**Fig. 1A:** MALP-A: S-[2,3-bispalmitoyloxy-(2RS)-propyl]cysteinyl-SKKKK.

**Fig. 1B:** N-palmitoyloxy-MALP-A: S-[2,3-bispalmitoyloxy-(2RS)-propyl]-N-palmitoylcysteinyl-SKKKK.

**Fig. 1C:** MALP-H: S-[2,3-bispalmitoyloxy-(2RS)-propyl]-cysteinyl-GQTNT.

**Fig. 1D:** S-MALP-2: S-[2,3-bispalmitoyloxy-(2S)-propyl]-cysteinyl-GNNDESNISFKEK; and R-MALP-2: S-[2,3-bispalmitoyloxy-(2R)-propyl]-cysteinyl-GNNDESNISFKEK.

With the help of this assay it is shown that the length or composition of the peptide part of the lipopeptides only has a small influence on the activity of macrophage stimulation (cf. **Fig. 1A, 1C and 1D**), whereas the number of fatty acids at the N-terminus (cf. **Fig. 1A and 1B**) and in particular the stereoconfiguration at the 2-position of the diacyloxypropyl group has an influence (cf. **Fig 1D**).

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**Example 2:**

The model of a flowing in of granulocytes and macrophages into the peritoneal cavity of a mouse is used as an example for the effectiveness of synthetic lipopeptides or liposomes in which such lipopeptides were incorporated. Mice of the NMRI-breed were used as test animals to exclude genetic variations.

The racemic lipopeptide MALP-2 was prepared according to Muehlradt et al. in (6), and compounds R-MALP-2 = S-[2,3-bispalmitoyloxy-(2R)-propyl]cysteinyl-GNNDESNISFKEK and S-MALP-2 = S-[2,3-bispalmitoyloxy-(2S)-propyl]cysteinyl-GNNDESNISFKEK were prepared according to quotation 7. MALP-containing liposomes are constructed as follows: The lipides (phosphatidylglycerin, phosphatidylserine, cholesterol, NBD-PE, molar ratio 1.08 : 1 : 0.25 : 0.005) dissolved in chloroform and chloroform/methanol (1 + 1), respectively, are pipetted together with MALP-2, dissolved in 2-propanol/H<sub>2</sub>O (1+1), and concentrated with a rotary evaporator. A complete drying of the lipide film is conducted over night in a sterile bench (Sterilbank). Dissolution of the dried lipid film in octylglucoside (100 mM in 0.05 M Tris-buffered NaCl, pH 7), 30 min. at 37 °C in a water bath. Dialysis against the 50-fold volume NaCl (0.1 M Tris-buffered, pH 7) at room temperature, 2 changes of the outer dialysate after 24 hours each.

The resulting liposome suspension is washed altogether 3 times with NaCl (centrifugation 30 min. at 47800 g, 4 °C) and thereafter resuspended in NaCl.

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At the point of time 0 the following preparations are injected sterile and in a physiological NaCl solution into the abdominal cavity of the test animals. Groups of 6 mice are killed after different periods; the abdominal cavity is rinsed with 1.2 ml sterile NaCl solution and the number of leucocytes and their composition is examined in this cell suspension.

**Figure 2** shows the flowing in of total leucocytes and granulocytes, respectively, as reaction to the intraperitoneal injection of 9 µg racemic MALP-2 in NMRI-mice (groups of 6 animals).

**Figure 3** shows the flowing in of total leucocytes and granulocytes, respectively, as reaction to the intraperitoneal injection of 0.2 mg liposomes which contained 9 µg MALP-2, in NMRI-mice (groups of 6 animals).

**Figure 4** shows the flowing in of total leucocytes and granulocytes as reaction to the intraperitoneal injection of 0.2 mg control liposomes, free of MALP-2, in NMRI-mice (groups of 6 animals).

**Figure 5A** shows the infiltration of leucocytes in the back-skin of a NMRI-mouse, 3 days after an intracutaneous injection of 2 µg S-MALP-2 which had been incorporated into liposomes.

**Figure 5B** (comparison): backskin of an untreated mouse.

**Figure 6** shows the area of new tissue with new vessels (middle of the figure) formed after a MALP injection.

**Table 1** summarizes the most important data, i. e. the increase of granulocytes and the later increase of macrophages after injecting the preparations. An about 100-fold increase of granulocytes compared to untreated control animals results, whereas the macrophages have increased 2-fold to 3-fold after 3 days.

If the chemotactically active chemokines MIP-1 $\alpha$ , MIP-2 or KC are measured 2 hours after an application of the mycoplasmas or lipopeptide preparations in serum of the test animals, activities can be found which are significantly increased.

**Example 3:**

**Table 2** shows the importance of the asymmetric C-atom at C2 of the dihydroxypropyl group for the in vivo effect. In this example as mentioned above, different amounts of R-MALP-2 = S-[2,3-bispalmitoyloxy-(2R)-propyl]cysteinyl-GNNDESNISFKEK and S-MALP-2 = S-[2,3-bispalmitoyloxy-(2S)-propyl]cysteinyl-GNNDESNISFKEK, respectively, were intraperitoneally applied to groups of 5 NMRI-mice; after 3 days the total number and the composition of peritoneal leucocytes were measured. S-MALP-2 is clearly more effective.

**Example 4:**

The following experiment was conducted to show the effect of intracutaneously applied MALP-2. When 2  $\mu$ g free S-MALP or 2  $\mu$ g S-MALP incorporated in 0.1 mg liposomes are injected intracutaneously in the skin of NMRI-mice, at the injection site a significant accumulation of leucocytes is formed after

3 days (cf. **Figures 5A and 5B**) and after 6 days new tissue and new vessels are formed (**Figure 6**). This shows that the preparations are effective in skin and that they are able to promote wound healing.

**Example 5:** Effects of the topical application of S-MALP-2 on the immigration of cells and wound healing in mice

To establish a dosage response (Dosisantwort) two types of experiments were conducted:

1. Determination of cell immigration and proliferation by measuring the nucleic acids in skin cut-outs after the intracutaneous (ic) injection of different doses of MALP-2. On day -1 the backs of C57BLKS/J mice were shaved and the hair was removed using "Veet".

On day 0 the animals were anaesthetized using Metofane; different doses of MALP-2, dissolved in 30 % 2-propanol in a volume of upto 10 µl, were injected (ic) into the backs. Corresponding volumes of the vehicle free of MALP-2 were injected into the back of the same mice at a different site. The animals were sacrificed 10 days later; cut-outs of the skin (diameter 0.8 cm) of the MALP-2 injection sites and the control injection sites were taken. The biopsies of skin were defatted by a 15 minute extraction using methanol, methanol/chloroform (1+1) and chloroform and hydrolyzed over night at 110 °C in 10 N HCl. The samples were dried, taken up in water, clarified by filtering through a sterile filter (0.2 µm); the nucleic acids were determined by measuring the absorption at 260 nm. 5 µg MALP-2 per injection site were found to be optimal. Using this dose the nucleic acid content of

the MALP-2 injection site was  $97 \pm 43\%$  higher than the corresponding control biopsy (4 animals per group).

2. The cell population and cell proliferation were determined in skin cut-outs (diameter 0.8 cm) of wounds which had been treated with different amounts of MALP-2. The backs of (C57BLKS/J-m+/+Lepr<sup>db</sup>) mice (diabetic) were shaved and the hair was removed using "Veet" for this experiment. The animals were anaesthetized using Metofane and their backs were disinfected using Braunol. Then circular skin segments (diameter 0.8 cm) were punched out of the back of each mouse. The wounds were covered by transparent Hydrofilm (Hartmann, Germany); a volume of 50  $\mu$ l MALP-2 or vehicle were injected into the wound (Wundbett) through the film. The animals were sacrificed after 10 days; the thin layer of cells which closed the wound was punched out (diameter 0.8 cm). The punched out samples were hydrolyzed and the nucleic acids were determined as indicated above. A dose of 5  $\mu$ g MALP-2 per wound was also found to be optimal in this model.

Using this dose  $349 \pm 24\,$   $\mu$ g nucleic acid per biopsy sample was determined in the animals treated with MALP-2, whereas  $142 \pm 22\,$   $\mu$ g was determined in the control samples (4 animals per group;  $P < 0.0001$ ).

A wound healing experiment was conducted as follows. On day -1 the backs of C57BLKS/J-m+/+Lepr<sup>db</sup> mice (diabetic) were shaved. On day 0 the animals were anaesthetized using ether and their backs were disinfected using Braunoderm. Then circular segments (diameter 1.3 cm) were cut out of the skin of the backs of each mouse. The edges of the wounds were treated with benzoin tinctura; 100  $\mu$ l of the MALP-2 preparation or

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the vehicle were given into each wound (10 mice in each treated group). The wounds were covered by transparent patches available from Tegaderm. Every day until day 4 100 µl of the preparation (content: 5 µg MALP-2) or of the vehicle preparation were injected into the wound (Wundbett) through the Tegaderm dressing or placed in the wound when the dressing was changed. The mice were kept separately in individual cages. Baytril (Bayer) was added to the drinking water to avoid infections (50 mg/l Enrofloxacin). The dressings were changed once a week during a short anaesthesia of ether during which the edges of the wounds were traced on microscopic slides. The traced regions were determined using a standard CCD camera and ScionImage software. The wounds were visually checked for infections; liquid samples of obviously infected wounds were tested on blood agar plates. Mice having infected wounds were excluded from the further tests. The data is given in **Figure 7** and shows a significant acceleration of wound healing in the animals treated with S-MALP-2.

**Example 6:** Increase in the survival rate of mice in a peritonitis model using MALP-2 pretreatment

Colon aszendens stent peritonitis is a new model for abdominal sepsis which can occur after surgery (9). The following experiments were conducted to assess the ability of MALP-2 to improve the survival rate in this model. 2 µg S-MALP-2 in 200 µl physiological sodium chloride solution or the same volume of physiological sodium chloride solution were injected intraperitoneally to groups of 12 to 18 C57BL/6 mice 2 or 4 days before surgery. Surgery was conducted using ether anaesthesia. The abdomen was disinfected with 70 % ethanol and was then opened by a cut in the median line (1 cm). A venous

catheter (16 gauge) was inserted into the lumen of the descending colon and attached by two stitches of a Ethilon suture (7/0). Subsequently the inner needle of the stent was removed, the catheter shortened to a few mm length and a small drop of faeces was pressed out of the catheter. Before closing the abdomen the peritoneal cavity was rinsed with 0.5 ml sodium chloride solution. None of the mice of the sodium chloride control group (18 per group) survived more than 90 h. 4 of the 12 animals treated with MALP-2 two days before surgery survived 150 h or longer, whereas 6 of the 12 animals treated with MALP-2 four days before surgery survived 120 h or longer. The difference in the survival rate was significant statistically ( $P < 0.001$  according to the log-Rank test).

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Table 1. Flowing in of leucocytes as a result of intraperitoneal injection of racemic MALP-2 after 72 hours

treatment	peritoncal cells		monocytes + macrophages	
	(x 10 <sup>6</sup> )		(x 10 <sup>6</sup> )	(%)
MALP-2 (9 µg)	11.2 ± 3.1 <sup>b</sup>		5 ± 1.2 <sup>b</sup>	47.4 ± 15.2
liposome encapsulated MALP-2 (9 µg)	8.5 ± 1.2 <sup>c</sup>		4.9 ± 0.9 <sup>c</sup>	57.6 ± 8.1 <sup>c</sup>
control liposomes	6.4 ± 1.9		2.9 ± 1.2	44.5 ± 6.8
NaCl (0.9 %)	5.8 ± 0.5		2.8 ± 0.4	47.9 ± 1.7

(2<sup>nd</sup> page of table 1)

lymphocytes		granulocytes	
(x 10 <sup>6</sup> )	(%)	(x 10 <sup>6</sup> )	(%)
3.5 ± 1.2	32 ± 11 <sup>b</sup>	2.6 ± 2.7	20.2 ± 13.2 <sup>b</sup>
2.9 ± 1	33.4 ± 9 <sup>c</sup>	0.8 ± 0.2 <sup>c</sup>	9.7 ± 2.2 <sup>c</sup>
3.2 ± 0.9	51.8 ± 6.7	0.2 ± 0.08	2.9 ± 1.4
2.8 ± 0.2	48.8 ± 1	0.05 ± 0.03	0.9 ± 0.6

Groups of 6 animals were used.

<sup>b</sup> significantly different ( $P < 0.05$ ) in comparison to control animals treated with NaCl solution (according to Student's t-test)

<sup>c</sup> significantly different ( $P < 0.05$ ) compared to animals treated with control liposomes (according to Student's t-test)

Table 2: Flowing in of leucocytes 72 hours after intraperitoneal injection of R-MALP-2 and S-MALP-2 resp.

		PEC	macrophages		lymphocytes	
		(x 10 <sup>6</sup> )	(x 10 <sup>6</sup> )	%	(x 10 <sup>6</sup> )	%
control	A	5,4	2,9	54	2,2	40,7
	B	6,4	3,8	58,6	2,4	38,1
	C	7,95	4,1	52,6	3,4	43,1
	D	7,5	2,9	38,5	4,4	57,9
	E	5,1	2,7	53,4	2,2	44
	ø	6,5 ± 1,3	3,3 ± 0,6	51,4 ± 7,6	2,9 ± 0,9	44,8 ± 7,7
S-MALP (10 µg)	A	11,1	4,5	40,1	6,3	56,5
	B	4,05	2,1	52,4	1,8	45,2
	C	11,4	6	53	5	44,2
	D	7,65	4	52,6	3,4	44,4
	E	9	4,6	51,3	4,1	45,6
	ø	8,64 ± 3	4,2 ± 1,4	49,9 ± 5,5	4,1 ± 1,7	47,2 ± 5,2
R-MALP (1 µg)	A	9,1	4,5	49,9	4,2	46,3
	B	10,8	4,7	43,8	5,2	48,3
	C	9,35	5,3	56,9	2,6	27,8
	D	13,7	8,4	61,6	4	29,4
	E	7,9	4,6	58,5	2,8	34,9
	ø	10,2 ± 2,2 <sup>a</sup>	5,5 ± 1,7 <sup>a</sup>	54,1 ± 7,2	3,8 ± 1,1	37,3 ± 9,5
R-MALP (5 µg)	A	14,4	6,3	44,1	5,1	35,2
	B	15,2	5,6	37,2	5,4	35,5
	C	17,3	6,1	34,8	10	58,1
	D	13,1	5,4	41,5	2,8	21,5
	E	12,5	6,3	50,7	4,2	33,4
	ø	14,5 ± 1,9 <sup>a,b</sup>	5,9 ± 0,4 <sup>a,b</sup>	41,7 ± 6,2	5,5 ± 2,7	36,7 ± 13,3

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neutrophiles	
(x 10 <sup>5</sup> )	%
0	0
0,19	0,3
0,24	0,3
0	0
0	0
$0,09 \pm 0,1$	$0,12 \pm 0,16$
0,56	0,5
0,32	0,8
0,34	0,3
0	0
0,27	0,3
$0,3 \pm 0,2$	$0,4 \pm 0,3$
0,46	0,5
3	2,8
12	12,8
10,1	7,4
2,8	3,5
$14,8 \pm$	$5,4 \pm 4,8^{\text{a,b}}$
$17,9^{\text{a,b}}$	
25,9	18
41	27
8,1	4,7
45,5	34,7
17,3	13,8
$27,6 \pm$	$19,6 \pm 11,6^{\text{a,b}}$
$15,7^{\text{a,b}}$	

(2<sup>nd</sup> page of table 2)<sup>a</sup> significant deviation from the control animals (without injection)<sup>b</sup> significant deviation from the animals treated with R-MALP (10 µg)